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UTILITY PATENT APPLICATION TRANSMITTAL

(Small Entity)

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Docket No.
ISPH-0519

Total Pages in this Submission

TO THE ASSISTANT COMMISSIONER FOR PATENTS

Box Patent Application
Washington, D.C. 20231

Transmitted herewith for filing under 35 U.S.C. 111(a) and 37 C.F.R. 1.53(b) is a new utility patent application for an invention entitled:

ANTISENSE MODULATION OF PI3K P85 EXPRESSION

and invented by:

Monia et al.

JC564 U.S. PTO
09/715983
11/20/00**If a CONTINUATION APPLICATION, check appropriate box and supply the requisite information:**

Continuation Divisional Continuation-in-part (CIP) of prior application No.: PCT/US00/40261

Which is a:

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Enclosed are:

Application Elements

1. Filing fee as calculated and transmitted as described below

2. Specification having 107 pages and including the following:
 - a. Descriptive Title of the Invention
 - b. Cross References to Related Applications (*if applicable*)
 - c. Statement Regarding Federally-sponsored Research/Development (*if applicable*)
 - d. Reference to Microfiche Appendix (*if applicable*)
 - e. Background of the Invention
 - f. Brief Summary of the Invention
 - g. Brief Description of the Drawings (*if drawings filed*)
 - h. Detailed Description
 - i. Claim(s) as Classified Below
 - j. Abstract of the Disclosure

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Application Elements (Continued)

3. Drawing(s) (*when necessary as prescribed by 35 USC 113*)
a. Formal b. Informal Number of Sheets _____
4. Oath or Declaration
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d. **DELETION OF INVENTOR(S)**
Signed statement attached deleting inventor(s) named in the prior application,
see 37 C.F.R. 1.63(d)(2) and 1.33(b).
5. Incorporation By Reference (*usable if Box 4b is checked*)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under
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6. Computer Program in Microfiche
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 - a. Paper Copy
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 - c. Statement Verifying Identical Paper and Computer Readable Copy

Accompanying Application Parts

8. Assignment Papers (*cover sheet & documents*)
9. 37 CFR 3.73(b) Statement (*when there is an assignee*)
10. English Translation Document (*if applicable*)
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Accompanying Application Parts (Continued)

15. Certified Copy of Priority Document(s) (*if foreign priority is claimed*)
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18. Pursuant to 35 U.S.C. 122(b)(2), Applicant hereby requests that this patent application not be published pursuant to 35 U.S.C. 122(b)(1). Applicant hereby certifies that the invention disclosed in this application has not and will not be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication of applications 18 months after filing of the application.

Warning

An applicant who makes a request not to publish, but who subsequently files in a foreign country or under a multilateral international agreement specified in 35 U.S.C. 122(b)(2)(B)(i), must notify the Director of such filing not later than 45 days after the date of the filing of such foreign or international application. A failure of the applicant to provide such notice within the prescribed period shall result in the application being regarded as abandoned, unless it is shown to the satisfaction of the Director that the delay in submitting the notice was unintentional.

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Fee Calculation and Transmittal

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For	#Filed	#Allowed	#Extra	Rate	Fee
Total Claims	63	- 20 =	43	x \$9.00	\$387.00
Indep. Claims	1	- 3 =	0	x \$40.00	\$0.00
Multiple Dependent Claims (check if applicable)					\$0.00
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Dated: November 20, 2000



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PATENT TRADEMARK OFFICE

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- 1) Patent Application Transmittal Letter (2 copies);
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Jane Massey Licata

JANE MASSEY LICATA

ANTISENSE MODULATION OF PI3K P85 EXPRESSION

This application is a continuation-in-part of PCT/US00/40261 filed June 21, 2000 which claims priority to US Application Serial No. 09/344,521 filed June 25, 1999, 5 now issued as U.S. Patent No. 6,100,090.

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of PI3K p85. In particular, this invention relates to antisense compounds, 10 particularly oligonucleotides, specifically hybridizable with nucleic acids encoding human PI3K p85. Such oligonucleotides have been shown to modulate the expression of PI3K p85.

BACKGROUND OF THE INVENTION

15 Many growth factors and hormones such as nerve growth factor (NGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF) and insulin mediate their signals through interactions with cell surface tyrosine kinase receptors. The transduction of extracellular 20 signals across the membrane, initiated by ligand binding, leads to the propagation of multiple signaling events which ultimately control target biochemical pathways within the cell.

The phosphatidylinositol 3-kinases (PI3Ks) represent a 25 ubiquitous family of heterodimeric lipid kinases that are found in association with the cytoplasmic domain of hormone

and growth factor receptors and oncogene products. PI3Ks act as downstream effectors of these receptors, are recruited upon receptor stimulation and mediate the activation of second messenger signaling pathways through
5 the production of phosphorylated derivatives of inositol (Fry, *Biochim. Biophys. Acta.*, **1994**, *1226*, 237-268).

PI3Ks have been implicated in many cellular activities including growth factor mediated cell transformation, mitogenesis, protein trafficking, cell survival and
10 proliferation, DNA synthesis, apoptosis, neurite outgrowth and insulin-stimulated glucose transport (reviewed in Fry, *Biochim. Biophys. Acta.*, **1994**, *1226*, 237-268).

The PI3 kinase enzyme heterodimers consist of a 110 kD (p110) catalytic subunit associated with an 85 kD (p85) regulatory subunit and it is through the SH2 domains of the p85 subunit that the enzyme associates with the membrane-bound receptors (Escobedo et al., *Cell*, **1991**, *65*, 75-82; Skolnik et al., *Cell*, **1991**, *65*, 83-90).

PI3K p85 (also known as GRB1 and PIK3R1) was initially isolated from bovine brain and shown to exist in two forms, α and β , encoded by two different genes. In these studies, both p85 isoforms were shown to bind to and act as substrates for tyrosine-phosphorylated receptor kinases and the polyoma virus middle T antigen complex (Otsu et al.,
25 *Cell*, **1991**, *65*, 91-104). The p85 α subunit has been shown to interact with other proteins including tyrosine kinase receptors such as the erythropoietin receptor, the PDGR- β receptor and Tie2, an endothelium-specific receptor involved in vascular development and tumor angiogenesis
30 (Escobedo et al., *Cell*, **1991**, *65*, 75-82; He et al., *Blood*, **1993**, *82*, 3530-3538; Kontos et al., *Mol. Cell. Biol.*, **1998**,

18, 4131-4140). It also interacts with focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase involved in integrin signaling and is thought to be a substrate and effector of FAK. Furthermore, the p85 α subunit also
5 interacts with profilin, an actin binding protein that facilitates actin polymerization (Bhargavi et al., *Biochem. Mol. Biol. Int.*, 1998, 46, 241-248; Chen and Guan, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, 91, 10148-10152), and the p85/profilin complex inhibits actin polymerization.

10 The murine homolog of PI3K p85 α gene has been isolated and characterized (Fruman, et al., *Genomics*, 1996, 37, 113-21). This gene was shown to produce alternative splice variants of 50, 55 and 85 kD each with unique expression patterns, the p50 α being the most abundant variant found in
15 liver. In addition, the novel splice variant, p55 α , has also been reported in rats [Shin, et al., *Biochem. Biophys. Res. Commun.*, 1998, 246, 313-319; Inukai, et al., *J. Biol. Chem.*, 1996, 271, 5317-20) and in humans (Antonetti, et al., *Moll. Cell. Biol.*, 1996, 16, 2195-203).

20 Characterization of this variant revealed its expression to be highest in brain and muscle. This variant, along with the full length p85 α variant, has been shown to interact with insulin receptor substrates and are thus likely to be involved in insulin and glucose mediated
25 signal transduction.

Recently, a truncated form of the PI3K p85 α subunit was isolated (Jimenez et al., *Embo J.*, 1998, 17, 743-753). This form includes the first 571 amino acids of the wild type (encoded by nucleotides 43-1755 of Genbank Acc. No.
30 M61906) linked to a region that is conserved in the eph

tyrosine kinase receptor family. This truncation was shown to induce the constitutive activation of PI3 kinase and contribute to cellular transformation of mammalian fibroblasts.

5 Terauchi et al. have generated mice with a targeted disruption of the gene encoding PI3K p85 α (Terauchi, et al., *Nat. Genet.*, 1999, 21, 230-5). These mice showed increased insulin sensitivity and hypoglycemia due to increased glucose transport in skeletal muscle and
10 adipocytes. Interestingly, the activity of PI3K associated with insulin receptor substrates (IRSs) was found to be mediated by the full-length p85 α in wild-type mice but by an alternative splice variant, p50 α , in the knockout mice.

Recently, mice with disruptions in all three splice
15 variants have been generated (Fruman, et al., *Science*, 1999, 283, 393-397). Most of these mice die within days after birth. Heterozygous mice, however, were studied and found to have impaired B-cell development and proliferation indicating that PI3K p85 α and its variants may play a role
20 in signal transduction pathways of the immune system.

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of PI3 kinase and the major approach to studying PI3 kinase function has been the use of chemical inhibitors, one phosphorothioate antisense
25 oligonucleotide targeted to the first 24 nucleotides of the coding sequence of the p85 α variant (Skorski, et al., *Blood*, 1995, 86, 726-36; Zauli, et al., *Blood*, 1997, 89, 883-95) and knockout mice.

Several chemically distinct inhibitors for PI3 kinases
30 are reported in the literature. These include wortmannin, a fungal metabolite (Ui et al., *Trends Biochem. Sci.*, 1995,

20, 303-307); demethoxyviridin, an antifungal agent
(Woscholski et al., *FEBS Lett.*, 1994, 342, 109-114) and
quercetin and LY294002, two related chromones (Vlahos et
al., *J. Biol. Chem.*, 1994, 269, 5241-5248). However, these
5 inhibitors primarily target the p110 subunit and are
untested as therapeutic protocols. Consequently, there
remains a long felt need for additional agents capable of
effectively inhibiting PI3K p85 α function.

Alternatively, antisense technology is emerging as an
10 effective means for reducing the expression of specific
gene products and may therefore prove to be uniquely useful
in a number of therapeutic, diagnostic, and research
applications for the modulation of PI3K p85 expression.

The present invention provides compositions and
15 methods for modulating PI3K p85 α expression, including
modulation of the truncated form of PI3K p85 α and the
splice variants of PI3K p85 α , p50 α and p55 α .

SUMMARY OF THE INVENTION

The present invention is directed to antisense
20 compounds, particularly oligonucleotides, which are
targeted to a nucleic acid encoding PI3K p85, and which
modulate the expression of PI3K p85. Pharmaceutical and
other compositions comprising the antisense compounds of
the invention are also provided. Further provided are
25 methods of modulating the expression of PI3K p85 in cells
or tissues comprising contacting said cells or tissues with
one or more of the antisense compounds or compositions of
the invention. Further provided are methods of treating an
animal, particularly a human, suspected of having or being
30 prone to a disease or condition associated with expression

of PI3K p85 by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding PI3K p85, ultimately modulating the amount of PI3K p85 produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding PI3K p85. As used herein, the terms "target nucleic acid" and "nucleic acid encoding PI3K p85" encompass DNA encoding PI3K p85, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of PI3K p85. In the context of the present invention, "modulation" means either an increase

(stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

5 It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose
10 function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is
15 a nucleic acid molecule encoding PI3K p85. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the
20 context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA
25 molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and
30 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and

"start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that 5 eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start 10 codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding PI3K p85, regardless of the sequence(s) of such codons.

15 It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and 20 "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination 25 codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which 30 is known in the art to refer to the region between the translation initiation codon and the translation

termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the 5 translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' 10 direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA 15 via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly 20 translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., 25 intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to 30 rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and

therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently 5 complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, 10 Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds.

"Complementary," as used herein, refers to the capacity for 15 precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to 20 each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" 25 and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense 30 compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An

antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides 5 composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms 10 because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form 15 of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 20 nucleobases (i.e. from about 8 to about 30 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 12 to about 25 nucleobases. As is known in the art, a nucleoside is a base-sugar combination. 25 The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of 30 the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to

either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this 5 linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The 10 normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural 15 internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as 20 sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, 25 phosphorodithioates, phosphotriesters, aminoalkyl-phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, 30 thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having

normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are 5 also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 10 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 15 5,563,253; 5,571,799; 5,587,361; and 5,625,050, certain of which are commonly owned with this application, and each of 15 which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or 20 cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone 25 backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S 30 and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of 10 which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an 15 appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is 20 replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the 25 preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, 1991, 254, 1497-1500.

30 Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and

oligonucleosides with heteroatom backbones, and in particular -CH₂-NH-O-CH₂- , -CH₂-N(CH₃)-O-CH₂- [known as a methylene (methylimino) or MMI backbone], -CH₂-O-N(CH₃)-CH₂- , -CH₂-N(CH₃)-N(CH₃)-CH₂- and -O-N(CH₃)-CH₂-CH₂- [wherein the 5 native phosphodiester backbone is represented as -O-P-O-CH₂-] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. 10 patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 15 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, 20 15 aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other 25 30 substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH₂CH₂OCH₃, also

known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoxyethoxy, i.e., a $\text{O}(\text{CH}_2)_2\text{ON}(\text{CH}_3)_2$ group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O- $\text{CH}_2-\text{O}-\text{CH}_2-\text{N}(\text{CH}_3)_2$, also described in examples hereinbelow.

Other preferred modifications include 2'-methoxy (2'-O- CH_3), 2'-aminopropoxy (2'- $\text{OCH}_2\text{CH}_2\text{CH}_2\text{NH}_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine

(C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by 20 Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for 25 increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been 30 shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds.,

Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

5 Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 10 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United 15 States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the 20 oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, 1989, 25 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, 1992, 660, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et 30 al., *Nucl. Acids Res.*, 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-

Behmoaras et al., *EMBO J.*, 1991, 10, 1111-1118; Kabanov et al., *FEBS Lett.*, 1990, 259, 327-330; Svinarchuk et al., *Biochimie*, 1993, 75, 49-54), a phospholipid, e.g., dihexadecyl-rac-glycerol or triethylammonium 1,2-di-O-
5 hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654; Shea et al., *Nucl. Acids Res.*, 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, 1995, 14, 969-973), or adamantine acetic acid
10 (Manoharan et al., *Tetrahedron Lett.*, 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, 1996, 277, 923-937).

15 Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802;
20 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506;
25 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the

instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than 5 one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds.

"Chimeric" antisense compounds or "chimeras," in the 10 context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically 15 contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide 20 may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing 25 the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target 30 region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary,

associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more
5 oligonucleotides, modified oligonucleotides,
oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid
10 structures include, but are not limited to, U.S.:
5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878;
5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355;
5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is
15 herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors
20 including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.
25

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.

30 The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other

molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative 5 United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 10 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

15 The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite 20 or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

25 The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the 30 oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to

the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to 5 physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are 10 formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, 15 diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a 20 sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms 25 somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an 30 acid form of one of the components of the compositions of the invention. These include organic or inorganic acid

salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and
5 include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for
10 example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid,
15 mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or
20 aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic
25 acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically
30 acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and

include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of PI3K p85 is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense

compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful
5 for research and diagnostics, because these compounds hybridize to nucleic acids encoding PI3K p85, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid
10 encoding PI3K p85 can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of PI3K p85 in a
15 sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered
20 in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or
25 insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or
30 infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at

least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, 5 ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

10 Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

15 Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and 20 other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but 25 are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional 30 techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association

the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided 5 solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, liquid syrups, soft 10 gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium 15 carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, 20 but not limited to, emulsions, microemulsions, creams, jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may 25 be applied to the formulation of the compositions of the present invention.

Emulsions

The compositions of the present invention may be 30 prepared and formulated as emulsions. Emulsions are

typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and

water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small
5 water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no
10 thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may
15 be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion.

Emulsifiers may broadly be classified into four categories:
20 synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y.,
30 volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), Marcel Dekker, Inc.,

New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the 5 hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and 10 amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, 15 lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers 20 especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium 25 aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, 30 fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants

(Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and

antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for
5 their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of
10 ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger
15 and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

20 In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable
25 liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and
30 then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a

transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules

5 (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant,

10 cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules

15 (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how

20 to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Bunker (Eds.), 1988, Marcel Dekker, Inc., New York,

25 N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

30 Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-

ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), 5 decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves 10 to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self- 15 emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not 20 limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils 25 and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral 30 bioavailability of drugs, including peptides
(Constantinides et al., *Pharmaceutical Research*, 1994, 11,

1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*,
1993, 13, 205). Microemulsions afford advantages of
improved drug solubilization, protection of drug from
enzymatic hydrolysis, possible enhancement of drug
5 absorption due to surfactant-induced alterations in
membrane fluidity and permeability, ease of preparation,
ease of oral administration over solid dosage forms,
improved clinical potency, and decreased toxicity
(Constantinides et al., *Pharmaceutical Research*, 1994, 11,
10 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138-143). Often
microemulsions may form spontaneously when their components
are brought together at ambient temperature. This may be
particularly advantageous when formulating thermolabile
drugs, peptides or oligonucleotides. Microemulsions have
15 also been effective in the transdermal delivery of active
components in both cosmetic and pharmaceutical
applications. It is expected that the microemulsion
compositions and formulations of the present invention will
facilitate the increased systemic absorption of
20 oligonucleotides and nucleic acids from the
gastrointestinal tract, as well as improve the local
cellular uptake of oligonucleotides and nucleic acids
within the gastrointestinal tract, vagina, buccal cavity
and other areas of administration.
25 Microemulsions of the present invention may also
contain additional components and additives such as
sorbitan monostearate (Grill 3), Labrasol, and penetration
enhancers to improve the properties of the formulation and
to enhance the absorption of the oligonucleotides and
30 nucleic acids of the present invention. Penetration
enhancers used in the microemulsions of the present

invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 5 1991, p. 92). Each of these classes has been discussed above.

Liposomes

There are many organized surfactant structures besides microemulsions that have been studied and used for the 10 formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term 15 "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the 20 composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

25 In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to 30 pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect
5 encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome
10 formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological
15 membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

20 Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-
25 effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

30 Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into

the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

5 Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized
10 in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, **1987**, 147, 980-985).

Liposomes which are pH-sensitive or
15 negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have
20 been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, **1992**, 19, 269-274).

One major type of liposomal composition includes
25 phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl
30 phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine

(DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

5 Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an
10 emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal
15 formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant
20 and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/
25 cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu et al.
S.T.P. Pharma. Sci., 1994, 4, 6, 466).

30 Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to

liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized 5 liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to 10 be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced 15 uptake into cells of the reticuloendothelial system (RES) (Allen et al., *FEBS Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993, 53, 3765). Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos et al. (*Ann. N.Y. Acad. Sci.*, 1987, 507, 20 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 25 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 30 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C₁₂15G, that contains a PEG moiety. Illum *et al.* (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klitanov *et al.* (*FEBS Lett.*, 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et*

al.) and in WO 94/20073 (Zalipsky et al.) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi et al.). U.S. Patent Nos. 5,540,935 (Miyazaki et al.) and 5,556,948 (Tagawa et al.) describe 5 PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry et al. discloses methods for encapsulating high molecular weight 10 nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa et al. discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman et al. describes certain methods of encapsulating 15 oligodeoxynucleotides in liposomes. WO 97/04787 to Love et al. discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive 20 candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, 25 e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal 30 composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of

serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such
5 as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also
10 known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is
15 classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic
20 esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block
25 polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant
30 is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of

amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important
5 members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include
10 quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is
15 classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in
20 *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York,
NY, 1988, p. 285).

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient
25 delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic
30 drugs may cross cell membranes if the membrane to be

crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

5 Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above
10 mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical
15 entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In
20 addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical
25 emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid),
30 myristic acid, palmitic acid, stearic acid, linoleic acid,

linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, 5 C₁₋₁₀ alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, 10 *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and 15 fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term 20 "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium 25 dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycolic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), 30 chenodeoxycholic acid (sodium chenodeoxycholate),

ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydrofusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; 5 Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25; 10 Yamashita et al., *J. Pharm. Sci.*, 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that 15 absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal 20 ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5- 25 methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi,

Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur et al., J. Control Rel., 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds
5 can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33*). This
10 class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems, 1991*, page 92); and non-steroidal anti-inflammatory agents such as diclofenac
15 sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, 1987, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example,
20 cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

25 Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a 5 nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active 10 nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of 15 nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, 20 dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyanostilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

Excipients

25 In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is 30 selected, with the planned manner of administration in

mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to,

5 binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen

10 phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrants (e.g., starch, sodium starch

15 glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be

20 used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin,

25 hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or

30 solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically

acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients 5 include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

10 Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the 15 compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage 20 forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the 25 compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic

substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for 5 example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other 10 chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, 15 cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUDR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual 20 of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 1206-1228). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, 25 acyclovir and ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49, respectively). Other non-antisense chemotherapeutic agents 30 are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds

5 targeted to a second nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the

10 skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules

15 can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and

20 can generally be estimated based on EC₅₀s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years.

25 Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy

30 to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses,

ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred 5 embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites for Oligonucleotide Synthesis

Deoxy and 2'-alkoxy amidites

5 2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein
10 incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds.

15 Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 25 1993, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-

arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine
5 was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl- (DMT) and 5'-DMT-3'
10 phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-
15 arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups.
20 Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was
25 accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard

procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via
5 amination of 2'-deoxy-2'-fluorouridine, followed by
selective protection to give N4-benzoyl-2'-deoxy-2'-
fluorocytidine. Standard procedures were used to obtain
the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

10 2'-O-Methoxyethyl-substituted nucleoside amidites are
prepared as follows, or alternatively, as per the methods
of Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

**2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-
methyluridine]**

15 5-Methyluridine (ribosylthymine, commercially
available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M),
diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate
(2.0 g, 0.024 M) were added to DMF (300 mL). The mixture
20 was heated to reflux, with stirring, allowing the evolved
carbon dioxide gas to be released in a controlled manner.
After 1 hour, the slightly darkened solution was
concentrated under reduced pressure. The resulting syrup
was poured into diethylether (2.5 L), with stirring. The
25 product formed a gum. The ether was decanted and the
residue was dissolved in a minimum amount of methanol (ca.
400 mL). The solution was poured into fresh ether (2.5 L)
to yield a stiff gum. The ether was decanted and the gum
was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to

give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or 5 it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-
10 methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol
(1.2 L) were added to a 2 L stainless steel pressure vessel
and placed in a pre-heated oil bath at 160°C. After heating
for 48 hours at 155-160°C, the vessel was opened and the
solution evaporated to dryness and triturated with MeOH
15 (200 mL). The residue was suspended in hot acetone (1 L).
The insoluble salts were filtered, washed with acetone (150
mL) and the filtrate evaporated. The residue (280 g) was
dissolved in CH₃CN (600 mL) and evaporated. A silica gel
column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3)
20 containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂
(250 mL) and adsorbed onto silica (150 g) prior to loading
onto the column. The product was eluted with the packing
solvent to give 160 g (63%) of product. Additional
material was obtained by reworking impure fractions.

25 **2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**
2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was
co-evaporated with pyridine (250 mL) and the dried residue
dissolved in pyridine (1.3 L). A first aliquot of
dimethoxytrityl chloride (94.3 g, 0.278 M) was added and

the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the 5 reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH₃CN (200 mL). The residue was dissolved in CHCl₃ (1.5 L) and extracted with 2x500 mL of saturated NaHCO₃ and 2x500 mL of saturated NaCl. The organic phase was dried over Na₂SO₄, 10 filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et₃NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional 15 was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine 20 (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the 25 addition of MeOH. Upon completion of the reaction, as judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl₃ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water 30 layers were back extracted with 200 mL of CHCl₃. The

combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were 5 evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-
10 acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl₃,
15 was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored
20 overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl,
25 dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5'-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5'-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyl-cytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO₄ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et₃NH as the

eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

5 N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting 10 mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were 15 combined, dried over MgSO₄ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

20 **2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside 25 amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected

with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

5'-O-tert-Butyldiphenylsilyl-2'-anhydro-5-methyluridine

5 O²-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml) at ambient temperature under an argon atmosphere and with mechanical stirring. *tert*-Butyldiphenylchlorosilane
10 (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned
15 between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the
20 solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether (3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

25 **5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine**

In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene

glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-Butyldiphenylsilyl-²-O-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were 5 added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for 10 ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped, concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions 15 used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl 20 acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and pure reusable starting material 20g. The yield based on starting material less pure recovered starting 25 material was 58%. TLC and NMR were consistent with 99% pure product.

2'-O-([2-phthalimidoxy]ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine

5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5- 30 methyluridine (20g, 36.98mmol) was mixed with

triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P₂O₅ under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop.

After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

5'-O-tert-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy)ethyl]-5-methyluridine

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butyldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH₂Cl₂ (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH₂Cl₂ and the combined organic phase was washed with water, brine and dried over anhydrous Na₂SO₄. The solution was concentrated to get 2'-O-(aminoxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting mixture was stirred for 1 h. Solvent was removed under

vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine

5 **5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinoxy) ethyl]-5-methyluridine** (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium 10 cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC 15 (5% MeOH in CH₂Cl₂). Aqueous NaHCO₃ solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na₂SO₄, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was 20 added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was 25 removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO₃ (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na₂SO₄ and evaporated to dryness . The residue obtained was 30 purified by flash column chromatography and eluted with 5%

MeOH in CH₂Cl₂ to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

2'-O-(dimethylaminoxyethyl)-5-methyluridine

5 Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and 10 stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH₂Cl₂). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH₂Cl₂ to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

15 **5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine**

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P₂O₅ under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH₂Cl₂ (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylamino-oxyethyl)-5-methyluridine (1.13g, 80%).

5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

5' -O-DMT-2' -O- (dimethylaminoxyethyl) -5-methyluridine
5 (1.08g, 1.67mmol) was co-evaporated with toluene (20mL).
To the residue N,N-diisopropylamine tetrazonide (0.29g,
1.67mmol) was added and dried over P₂O₅ under high vacuum
overnight at 40°C. Then the reaction mixture was dissolved
in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-
10 N,N,N¹,N¹-tetraisopropylphosphoramidite (2.12mL, 6.08mmol)
was added. The reaction mixture was stirred at ambient
temperature for 4 hrs under inert atmosphere. The progress
of the reaction was monitored by TLC (hexane:ethyl acetate
1:1). The solvent was evaporated, then the residue was
15 dissolved in ethyl acetate (70mL) and washed with 5%
aqueous NaHCO₃ (40mL). Ethyl acetate layer was dried over
anhydrous Na₂SO₄ and concentrated. Residue obtained was
chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-
2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-(2-
20 cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam
(1.04g, 74.9%).

2'-(Aminooxyethoxy) nucleoside amidites

2'-(Aminooxyethoxy) nucleoside amidites [also known in
the art as 2'-O-(aminooxyethyl) nucleoside amidites] are
25 prepared as described in the following paragraphs.
Adenosine, cytidine and thymidine nucleoside amidites are
prepared similarly.

N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite]

The 2'-O-aminoxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinocco, C. J., WO 94/02501 A1 940203.) Standard protection procedures should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxypthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH₂-O-CH₂-N(CH₃)₂, or 2'-DMAEOE nucleoside amidites) are

prepared as follows. Other nucleoside amidites are prepared similarly.

2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

2 [2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O²⁻, 2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and

extracted with CH₂Cl₂ (2x200 mL). The combined CH₂Cl₂ layers are washed with saturated NaHCO₃ solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH₂Cl₂:Et₃N (20:1, v/v, with 1% triethylamine) gives the title compound.

5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH₂Cl₂ (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

Example 2

20 Oligonucleotide synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation

wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating 5 twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as 10 described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

15 Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and 20 PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

25 Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both 30 herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedi-
5 methylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleo-
10 sides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

15 Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by
20 reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to
25 in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be
5 of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of
10 the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gappers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me] -- [2'-deoxy] -- [2'-O-Me] Chimeric

15 **Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above.
20 Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait
25 step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then
30 lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect

all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced
5 to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)] -- [2'-deoxy] -- [2'-O-
10 (Methoxyethyl)] Chimeric Phosphorothioate
Oligonucleotides

[2'-O-(2-methoxyethyl)] -- [2'-deoxy] -- [-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl
15 chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl) Phosphodiester] -- [2'-deoxy
Phosphorothioate] -- [2'-O-(2-Methoxyethyl)
20 Phosphodiester] Chimeric Oligonucleotides
[2'-O-(2-methoxyethyl phosphodiester] -- [2'-deoxy phosphorothioate] -- [2'-O-(methoxyethyl) phosphodiester]
chimeric oligonucleotides are prepared as per the above
procedure for the 2'-O-methyl chimeric oligonucleotide with
the substitution of 2'-O-(methoxyethyl) amidites for the
25 2'-O-methyl amidites, oxidization with iodine to generate
the phosphodiester internucleotide linkages within the
wing portions of the chimeric structures and sulfurization
utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage

Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides
5 are synthesized according to United States patent
5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column
10 (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by
15 polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some
20 studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, 266, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

25 **Example 7**

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences

simultaneously in a standard 96 well format.

Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate

internucleotide linkages were generated by sulfurization

5 utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage

Reagent) in anhydrous acetonitrile. Standard base-

protected beta-cyanoethyldiisopropyl phosphoramidites were

purchased from commercial vendors (e.g. PE-Applied

Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ).

10 Non-standard nucleosides are synthesized as per known

literature or patented methods. They are utilized as base

protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides were cleaved from support and

deprotected with concentrated NH₄OH at elevated temperature

15 (55-60°C) for 12-16 hours and the released product then

dried in vacuo. The dried product was then re-suspended in

sterile water to afford a master plate from which all

analytical and test plate samples are then diluted

utilizing robotic pipettors.

20 **Example 8**

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption

spectroscopy. The full-length integrity of the individual

25 products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACE™ MDQ) or, for

individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone

composition was confirmed by mass analysis of the compounds

30 utilizing electrospray-mass spectroscopy. All assay test

plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

5 **Example 9**

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at 10 measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

T-24 cells:

15 The transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal 20 calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were 25 seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates

and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained
5 from the American Type Culture Collection (ATCC) (Manassas,
VA). A549 cells were routinely cultured in DMEM basal
media (Gibco/Life Technologies, Gaithersburg, MD)
supplemented with 10% fetal calf serum (Gibco/Life
Technologies, Gaithersburg, MD), penicillin 100 units per
10 mL, and streptomycin 100 micrograms per mL (Gibco/Life
Technologies, Gaithersburg, MD). Cells were routinely
passaged by trypsinization and dilution when they reached
90% confluence.

NHDF cells:

15 Human neonatal dermal fibroblast (NHDF) were obtained
from the Clonetics Corporation (Walkersville MD). NHDFs
were routinely maintained in Fibroblast Growth Medium
(Clonetics Corporation, Walkersville MD) supplemented as
recommended by the supplier. Cells were maintained for up
20 to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from
the Clonetics Corporation (Walkersville MD). HEKs were
routinely maintained in Keratinocyte Growth Medium
25 (Clonetics Corporation, Walkersville MD) formulated as
recommended by the supplier. Cells were routinely
maintained for up to 10 passages as recommended by the
supplier.

AML12 cells:

Alpha mouse liver 12 cells (AML12) were obtained from American Type Culture Collection (ATCC) (Manassas, VA). AML12 cells were routinely cultured in D-MEM/F-12 media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with Insulin/transferrin/selenium supplement (Gibco/Life Technologies, Gaithersburg, MD), 40 ng/ml dexamethasone (Sigma) penicillin-streptomycin (Gibco/Life Technologies, Gaithersburg, MD) and 10% fetal bovine serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 80% confluence.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200 μ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130 μ L of OPTI-MEM™-1 containing 3.75 μ g/mL LIPOFECTIN™ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

Example 10

**Analysis of oligonucleotide inhibition of PI3K p85
25 expression**

Antisense modulation of PI3K p85 expression can be assayed in a variety of ways known in the art. For example, PI3K p85 mRNA levels can be quantitated by, e.g.,

Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A) + mRNA.

- 5 Methods of RNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Northern blot analysis is routine in the art and is taught in, for example, Ausubel,
- 10 F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7700 Sequence Detection System, available from PE-Applied Biosystems,
- 15 Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

PI3K p85 protein levels can be quantitated in a variety of ways well known in the art, such as

20 immunoprecipitation, Western blot analysis (immunoblotting), ELISA or fluorescence-activated cell sorting (FACS). Antibodies directed to PI3K p85 can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation,

25 Birmingham, MI), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997.

30 Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in*

Molecular Biology, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al.,

- 5 *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

15 **Example 11**

Poly(A)+ mRNA isolation

- Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, 20 Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 60 µL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc.,

Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove 5 excess wash buffer and then air-dried for 5 minutes. 60 μ L of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

10 Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

15 Total mRNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μ L cold
20 PBS. 100 μ L Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 μ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a
25 QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of
30 the RNEASY 96™ plate and the vacuum applied for a period of

15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the
5 QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.
10 The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are
15 carried out.

Example 13

Real-time Quantitative PCR Analysis of PI3K p85 mRNA Levels

Quantitation of PI3K p85 mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700
20 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As
25 opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically
30 between the forward and reverse PCR primers, and contains

two fluorescent dyes. A reporter dye (e.g., JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, 5 obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of 10 the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of 15 the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics 20 built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide 25 treatment of test samples.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 µL PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM MgCl₂, 300 µM each of dATP, dCTP and dGTP, 600 µM of dUTP, 30 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD™, and

12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 μ L poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C.

Following a 10 minute incubation at 95°C to activate the 5 AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension). PI3K p85 probes and primers were designed to hybridize to the human PI3K p85 sequence, using published sequence information (GenBank 10 accession number M61906, incorporated herein as SEQ ID NO:1).

For PI3K p85 the PCR primers were:

forward primer: AGCAACCTGGCAGAATTACGA (SEQ ID NO: 2)
reverse primer: CAAACACGTGCACATCGATCAT (SEQ ID NO: 3) and
15 the PCR probe was: FAM-TTCTTGATTGTGATACACCCTCCGTGGACT-TAMRA (SEQ ID NO: 4) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH the PCR primers were:

20 forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 5)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO: 6) and the
PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCC- TAMRA 3' (SEQ
ID NO: 7) where JOE (PE-Applied Biosystems, Foster City,
CA) is the fluorescent reporter dye and TAMRA (PE-Applied
25 Biosystems, Foster City, CA) is the quencher dye.

Example 14

Northern blot analysis of PI3K p85 mRNA levels

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 30 mL RAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA

was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. 5 Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV 10 visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA).

Membranes were probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's 15 recommendations for stringent conditions with a PI3K p85 specific probe prepared by PCR using the forward primer AGCAACCTGGCAGAATTACGA (SEQ ID NO: 2) and the reverse primer CAAAACGTGCACATCGATCAT (SEQ ID NO: 3). To normalize for variations in loading and transfer efficiency membranes 20 were stripped and probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA). Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH 25 levels in untreated controls.

Example 15

**Antisense inhibition of PI3K p85 expression-
phosphorothioate oligodeoxynucleotides**

In accordance with the present invention, a series of 30 oligonucleotides were designed to target different regions

of the human PI3K p85 RNA, using published sequences (GenBank accession number M61906, incorporated herein as SEQ ID NO: 1). The oligonucleotides are shown in Table 1. Target sites are indicated by nucleotide numbers, as given 5 in the sequence source reference (Genbank accession no. M61906), to which the oligonucleotide binds. All compounds in Table 1 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on PI3K p85 mRNA levels 10 by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

15 Inhibition of PI3K p85 mRNA levels by phosphorothioate
oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
27977	Coding	88	atcttcttctctttcctt	0	8
27978	Coding	168	gcttcctgtccatcactg	29	9
27979	Coding	445	ttcaatggcttccacgag	6	10
20 27980	Coding	507	aattctgccagggttgctg	0	11
27981	Coding	605	gtaagtccaggagatgc	14	12
27982	Coding	642	atttcactgtaaacggct	19	13
27983	Coding	773	gcttgaagaaatgtttta	0	14
27984	Coding	859	ggctgctgagaatctgaa	22	15
25 27985	Coding	926	gttcattccattcagttg	13	16
27986	Coding	970	agtaggtttgggtgttt	0	17
27987	Coding	996	ttattcataaccgttgtt	4	18
27988	Coding	1022	attcagcattttaagg	1	19
27989	Coding	1230	accacagaactgaaggtt	0	20
30 27990	Coding	1455	atttcctggatgtgcgg	52	21
27991	Coding	1534	ccgctttgggtctggca	71	22
27992	Coding	1582	tttctcattgccttcacg	0	23
27993	Coding	1596	atcctttgtattttttc	15	24
27994	Coding	1674	ttcaagtcttcttccaat	15	25
35 27995	Coding	1763	attgggtctctcgctttc	0	26

27996	Coding	1808	tcaacttctttgccgaa	38	27	
27997	Coding	1824	ttgcccAACCAACTCGTTC	55	28	
27998	Coding	1840	gtcttcAGTGTTCATT	0	29	
27999	Coding	1925	CTTGTTCGGTTGCTGC	36	30	
5	28000	Coding	cctgtttactgctctccc	20	31	
28001	Coding	2015	ccaccacTACAGAGCAGG	0	32	
28002	Coding	2029	CTTACTCGCCGTCCAC	41	33	
28003	Coding	2068	aaAGCCATAGCCAGTTGC	0	34	
28004	Coding	2160	acattgagggAGTCGTTG	0	35	
10	28005	3'UTR	2265	gccctttgtttccagag	0	36
28006	3'UTR	2281	atcagactggagaggagc	27	37	
28007	3'UTR	2426	aaAGAAAGGGATAAGCACT	7	38	
28008	3'UTR	2605	ctgcctctctctccctccg	0	39	
28009	3'UTR	2651	ccaggctaaaccaggctg	57	40	
15	28010	3'UTR	2679	tgtctgggtccaccgtgc	55	41
28011	3'UTR	2741	gacgtgccttctgctac	28	42	
28012	3'UTR	2768	attctcccaaAGCGTCCC	19	43	
28013	3'UTR	2817	ttctggcactttctatga	28	44	
28014	3'UTR	2989	ccttcAGCAAAACAAAAC	24	45	
20	28015	3'UTR	3043	aactgaaataacaactta	6	46
28016	3'UTR	3294	ccaaacaaaacagtccaaa	6	47	

As shown in Table 1, SEQ ID NOS 21, 22, 27, 28, 30, 33, 40 and 41 demonstrated at least 30% inhibition of PI3K p85 expression in this assay and are therefore preferred.

25 **Example 16:**

Antisense inhibition of PI3K p85 expression-phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human PI3K p85 were 30 synthesized. The oligonucleotide sequences are shown in Table 2. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M61906), to which the oligonucleotide binds.

All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-
5 nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

10 Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from two experiments. If present, "N.D." indicates "no data".

Table 2

Inhibition of PI3K p85 mRNA levels by chimeric
15 phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
28017	Coding	88	atcttcttctttcctt	59	8
28018	Coding	168	gcttcctgtccatcactg	47	9
20	28019	Coding	445	ttcaatggcttccacgag	6
	28020	Coding	507	aattctgccagggtgctg	12
	28021	Coding	605	gtaagtccaggagatagc	43
	28022	Coding	642	atttcactgtaaacggct	69
	28023	Coding	773	gcttgaagaaaatgtttta	43
25	28024	Coding	859	ggctgctgagaatctgaa	59
	28025	Coding	926	gttcattccattcagttg	22
	28026	Coding	970	agttagtttggtggttt	54
	28027	Coding	996	ttattcataccgttgttg	45
	28028	Coding	1022	attcagcattttgtaagg	0
30	28029	Coding	1230	accacagaactgaaggtt	57
	28030	Coding	1455	atttcctggatgtgcgg	74
	28031	Coding	1534	ccgctctgggtctggca	15
	28032	Coding	1582	tttctcattgccttcacg	35

28033	Coding	1596	atcctttgtatttcttc	46	24	
28034	Coding	1674	ttcaagtcttcttccat	28	25	
28035	Coding	1763	attggctctcgctttc	0	26	
28036	Coding	1808	tcaacttctttgccgaa	59	27	
5	28037	Coding	1824	ttgcccaccactcgttc	28	28
28038	Coding	1840	gtcttcagtgtttcatt	0	29	
28039	Coding	1925	ctttgttgcgttgc	46	30	
28040	Coding	1988	cctgtttactgctctccc	0	31	
28041	Coding	2015	ccaccactacagagcagg	38	32	
10	28042	Coding	2029	ctttacttcgcgtccac	0	33
28043	Coding	2068	aaagccatagccagttgc	10	34	
28044	Coding	2160	acattgagggagtcgttg	0	35	
28045	3' UTR	2265	gccctttgtttccagag	0	36	
28046	3' UTR	2281	atcagactggagaggagc	32	37	
15	28047	3' UTR	2426	aaagaaggataaagcact	18	38
28048	3' UTR	2605	ctgcctctctctccctccg	0	39	
28049	3' UTR	2651	ccaggctaaaccaggctg	24	40	
28050	3' UTR	2679	tgtctgggtccaccgtgc	57	41	
28051	3' UTR	2741	gacgtgccttctgctac	53	42	
20	28052	3' UTR	2768	attctcccaaagcgtccc	55	43
28053	3' UTR	2817	ttctggcactttctatga	0	44	
28054	3' UTR	2989	ccttcagcaaaacaaaac	0	45	
28055	3' UTR	3043	aactgaaaataacaactta	14	46	
28056	3' UTR	3294	ccaacaaaacagtccaaa	15	47	

25 As shown in Table 2, SEQ ID NOS 8, 9, 12, 13, 14, 15,
17, 18, 20, 21, 23, 24, 27, 30, 32, 37, 41, 42 and 43
demonstrated at least 30% inhibition of PI3K p85 expression
in this experiment and are therefore preferred.

Example 17

30 Western blot analysis of PI3K p85 protein levels

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for

1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to PI3K p85 is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

Example 18

Antisense inhibition of mouse PI3K p85 expression-phosphorothioate 2'-MOE gapmer oligonucleotides

10 In accordance with the present invention, a series of oligonucleotides targeted to mouse PI3K p85 were synthesized. The oligonucleotide sequences are shown in Table 3. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. U50413; SEQ ID NO: 48), to which the oligonucleotide binds.

All compounds in Table 3 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which 20 is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues are 5-methylcytidines throughout the oligonucleotides.

Data were obtained by real-time quantitative RT-PCR as described in other examples herein and are averaged from two experiments. For mouse PI3K p85 the PCR primers were: forward primer: GCGTGGCAGTAAAATCAGACG (SEQ ID NO: 49)

reverse primer: CCACGTGTCCTTCTCAGCAA (SEQ ID NO: 50) and the PCR probe was: FAM- TGGGCCTCGCTGCGAGAGTCAG-TAMRA (SEQ ID NO: 51) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. If present, "N.D." indicates "no data".

Table 3

Inhibition of mouse PI3K p85 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

10

	ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhib.	SEQ ID NO.
	131406	3'UTR	9	cgtcgcttcctccaactcg	56	52
	131407	3'UTR	195	cgtccactctcagcttac	85	53
	131408	3'UTR	378	ccatctgtcctccatcaacg	88	54
15	131409	3'UTR	563	gcactcatgtctgcagct	85	55
	131410	Coding	694	cctggccatcactgaatcca	95	56
	131411	Coding	746	ccagtggtttcattgttagcc	90	57
	131412	Coding	889	cttgctgctccgtgtcagct	85	58
	131413	Coding	1081	tctccaagtccactgacg	56	59
20	131414	Coding	1130	tcggcgagatagcgttgaa	73	60
	131415	Coding	1281	atactgaagcgtaa	75	61
	131416	Coding	1473	tgctggtgctggctgtct	68	62
	131417	Coding	1670	ggtgtaa	78	63
	131418	Coding	1855	cctgctgtattggacact	50	64
25	131419	Coding	2062	gctccctgggttggcattgt	45	65
	131420	Coding	2233	cgtcttcgtactcagct	79	66
	131421	Coding	2439	gctcccgcacattccacgt	65	67
	131422	Coding	2594	ccatagccggtgccagtct	40	68
	131423	5'UTR	2790	tttgcttcagaggcctt	40	69
30	131424	5'UTR	3150	ggtctccaaagtccaaactt	N.D.	70
	131425	5'UTR	3241	gtctgggttaccacaccca	N.D.	71
	131426	5'UTR	3339	gcatcaatgttctctcaaag	75	72

As shown in Table 3, SEQ ID NOS 53, 54, 55, 56, 57, 58, 60
61, 62, 63, 66, 67, and 72 demonstrated at least 60%
35 inhibition of mouse PI3K p85 expression in this experiment
and are therefore preferred.

Example 19

Effects of antisense inhibition of mouse PI3K p85 (ISIS 131410) on mRNA expression in liver and fat

Leptin, the product of the obese gene, is a
5 circulating hormone secreted primarily from adipocytes and
which interacts with receptors in the hypothalamus to
inhibit eating. The lack of leptin in ob/ob mice, who are
homozygous for the obese gene, results in hyperglycemia,
hyperinsulinemia, hyperphagia, obesity, infertility,
10 decreased brain size and decreased stature. The importance
of this single peptide is demonstrated by the profound
obesity exhibited by the ob/ob mouse which is unable to
produce functional leptin.

Ob/ob mice are used as a model of obesity. The ob/ob
15 phenotype is due to a mutation in the leptin gene on a
C57BL/6J-Lep(ob) background. Heterozygous ob/wt mice (known
as lean littermates) do not display the
hyperglycemia/hyperlipidemia or obesity phenotype and,
along with wild-type mice, are used as controls.

20 In accordance with the present invention, the effects
of ISIS 131410 (SEQ ID NO: 56) on PI3K p85 mRNA expression
was investigated in the ob/ob mouse model of obesity.

Male ob/ob mice (age 9 weeks at time 0) were divided
into matched groups with the same average blood glucose
25 levels and treated by intraperitoneal injection once a week
with ISIS 141925 (GCCACCGCCTATGTCTTCTC; SEQ ID NO: 73; the
control oligonucleotide) or ISIS 131410. Mice were treated
at a dose of 25 mg/kg of ISIS 141925 or 25 mg/kg of ISIS
131410.

Treatment was continued for two weeks after which the mice were sacrificed and tissues collected for mRNA analysis. RNA values were normalized and are expressed as a percentage of saline treated control.

5 ISIS 131410 successfully reduced PI3K p85 mRNA levels in the liver and fat of ob/ob mice (to 52% and 55% of control, respectively), whereas the control treated animals showed no reduction in PI3K p85 mRNA, remaining at the level of the saline treated control.

10 Lean littermates (ob/wt) were also examined for mRNA reduction of PI3K p85 in the liver at doses of 25 and 50 mg/kg of ISIS 131410 or saline treatment. In these animals, at both doses, the level of expression was reduced only minimally to 80% of control.

15 **Example 20**

Effects of antisense inhibition of mouse PI3K p85 (ISIS 131410) on levels of p85 splice variant

ISIS 131410 is one of several antisense oligonucleotides of the present invention that hybridize to
20 the longer p85 α splice variant and not to the p55 α or the p50 α splice variant. Studies were therefore designed to study the effects of this antisense oligonucleotide on expression product of PI3K p85 α splice variant.

Analysis of the expression of the various splice
25 variants of PI3K p85 by immunoprecipitation with p110 (the catalytic subunit) and Western blot detection using the p85pan antibody (which recognizes all three variants) revealed that, in the livers of both ob/ob and wild-type mice, treatment with ISIS 131410 alters the species of PI3K
30 p85 variant present in favor of the p50 α variant.

Example 21

Effects of antisense inhibition of PI3 kinase p85 (ISIS 131410) on blood glucose levels

Male ob/ob and wild-type mice were divided into
5 matched groups with the same average blood glucose levels
and treated by intraperitoneal injection once a week with
saline, ISIS 131410 or the scrambled control, ISIS 141925.

Ob/ob mice were treated with saline, or doses of 25 or
50 mg/kg of ISIS 313410 (n=4) or ISIS 141925 (n=2) while
10 wild-type mice (n=3) were treated with saline or doses of
25 or 50 mg/kg of ISIS 131410. Treatment was continued for
two weeks with blood glucose levels being measured on day
0, 7 and 14.

By day 14 in ob/ob mice, blood glucose levels were
15 reduced at all doses of ISIS 131410 from a starting level
of 250 mg/dL at day 1 to 180 mg/dL at day 7 and 150 mg/dL
at day 14. These final levels are within the normal range
for wild-type mice (170 mg/dL). The scrambled control and
saline treated levels were 240 mg/dL and 250 mg/dL at day
20 14, respectively.

In wild-type mice, blood glucose levels remained
constant throughout the study for all treatment groups
(average 150 mg/dL). These results indicate that treatment
with ISIS 131410 reduces blood glucose in ob/ob mice and
25 that there is no hypoglycemia induced in the ob/ob or the
wild-type mice as a result of the oligonucleotide
treatment.

Example 22

Effects of antisense inhibition of mouse PI3K p85 (ISIS 131410) on serum insulin levels

Male ob/ob mice (age 9 weeks at time 0) were divided
5 into matched groups with the same average blood glucose
levels and treated by intraperitoneal injection once a week
with saline, ISIS 141925 (the control oligonucleotide) or
ISIS 131410 at a dose of 50 mg/kg. Treatment was continued
for two weeks with serum insulin levels being measured on
10 day 14.

Mice treated with ISIS 131410 showed a decrease in
serum insulin levels (5 ng/mL) compared to saline treated
animals (26 ng/mL) and control treated animals (28 ng/mL).

Collectively, these data show that antisense
15 oligonucleotides to PI3K p85 act to reduce serum insulin
and blood glucose *in vivo* and suggest that they have
potential therapeutic value in the treatment of disorders
associated with insulin and glucose regulation.

What is claimed is:

1. An antisense compound 8 to 30 nucleobases in length targeted to a nucleic acid molecule encoding PI3K p85, wherein said antisense compound specifically hybridizes with and inhibits the expression of PI3K p85.

2. The antisense compound of claim 1 which is an antisense oligonucleotide.

3. The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 21, 22, 27, 28, 30, 33, 40, 41, 8, 9, 12, 13, 14, 15, 17, 18, 20, 23, 24, 32, 37, 42, 43, 53, 54, 55, 56, 57, 58, 60, 61, 62, 63, 66, 67 or 72.

4. The antisense compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 21, 27, 30 or 41.

5. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

6. The antisense compound of claim 5 wherein the modified internucleoside linkage is a phosphorothioate linkage.

7. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

8. The antisense compound of claim 7 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

9. The antisense compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

10. The antisense compound of claim 9 wherein the modified nucleobase is a 5-methylcytosine.

11. The antisense compound of claim 1 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

12. A pharmaceutical composition comprising the antisense compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The pharmaceutical composition of claim 12 further comprising a colloidal dispersion system.

14. The pharmaceutical composition of claim 12 wherein the antisense compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of PI3K p85 in human cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 1 so that expression of PI3K p85 is inhibited.

16. A method of treating a human having a disease or condition associated with PI3K p85 comprising administering to said human a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that expression of PI3K p85 is inhibited.

17. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

18. The method of claim 17 wherein the hyperproliferative disorder is cancer.

19. The method of claim 16 wherein the disease or condition is a metabolic disease or condition.

20. The method of claim 16 wherein the disease or condition is diabetes.

21. The method of claim 16 wherein the disease or condition is Type 2 diabetes.

22. The method of claim 16 wherein the disease or condition is obesity.

23. The antisense compound of claim 1 which is targeted to a nucleic acid molecule encoding a truncated form of human PI3K p85.

24. A method of decreasing blood glucose levels in an animal comprising administering to said animal the antisense compound of claim 1.

25. The method of claim 24 wherein the animal is a human or a rodent.

26. The method of claim 24 wherein the blood glucose levels are plasma glucose levels or serum glucose levels.

27. The method of claim 24 wherein the animal is a diabetic animal.

28. A method of decreasing insulin levels in an animal comprising administering to said animal the antisense compound of claim 1.

29. The method of claim 28 wherein the animal is a human or a rodent.

30. The method of claim 28 wherein the insulin levels are plasma insulin levels or serum insulin levels.

31. The method of claim 28 wherein the animal is a diabetic animal.

32. A method of preventing or delaying the onset of a disease or condition associated with PI3K P85 in an animal comprising administering to said animal a therapeutically or prophylactically effective amount of the antisense compound of claim 1.

33. The method of claim 32 wherein the animal is a human.

34. The method of claim 32 wherein the disease or condition is a metabolic disease or condition.

35. The method of claim 32 wherein the disease or condition is diabetes.

36. The method of claim 32 wherein the disease or condition is Type 2 diabetes.

37. The method of claim 32 wherein the disease or condition is obesity.

38. The method of claim 32 wherein the disease or condition is a hyperproliferative condition.

39. The method of claim 38 wherein the hyperproliferative condition is cancer.

40. A method of preventing or delaying the onset of an increase in blood glucose levels in an animal comprising administering to said animal the antisense compound of claim 1.

41. The method of claim 40 wherein the animal is a human or a rodent.

42. The method of claim 40 wherein the blood glucose levels are plasma glucose levels or serum glucose levels.

43. The method of claim 40 wherein the animal is a diabetic animal.

44. A method of preventing or delaying the onset of an increase in insulin levels in an animal comprising administering to said animal the antisense compound of claim 1.

45. The method of claim 44 wherein the animal is a human or a rodent.

46. The method of claim 44 wherein the insulin levels are plasma insulin levels or serum insulin levels.

47. The method of claim 44 wherein the animal is a diabetic animal.

48. The antisense compound of claim 1 which is targeted to PI3K p85a and which preferentially inhibits the expression of PI3K p85a.

49. The antisense compound of claim 1 which is targeted to a region of a nucleic acid molecule encoding PI3K p85a which is not found in a nucleic acid molecule encoding PI3K p50a.

50. The antisense compound of claim 1 which is targeted to a region of a nucleic acid molecule encoding PI3K p85a which is not found in a nucleic acid molecule encoding PI3K p55a.

51. The antisense compound of claim 1 which inhibits the expression of all splice variants encoded by the PI3K p85a gene.

52. The antisense compound of claim 1 which alters the ratio of PI3K p85a to PI3K p50a expressed by a cell or tissue.

53. The antisense compound of claim 48 which increases the ratio of PI3K p85a to PI3K p50a expressed by a cell or tissue.

54. The antisense compound of claim 48 which decreases the ratio of PI3K p85a to PI3K p50a expressed by a cell or tissue.

55. The antisense compound of claim 1 which alters the ratio of PI3K p85a to PI3K p55a expressed by a cell or tissue.

56. The antisense compound of claim 51 which increases the ratio of PI3K p85a to PI3K p55a expressed by a cell or tissue.

57. The antisense compound of claim 51 which decreases the ratio of PI3K p85a to PI3K p55a expressed by a cell or tissue.

58. A method of modulating PI3K signal transduction in cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 1 so that PI3K signal transduction is modulated.

59. A method of altering the ratio of PI3K p85a to PI3K 50a in human cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 48 so that the ratio of PI3K p85a to PI3K p50a is altered.

60. A method of altering the ratio of PI3K p85a to PI3K 55a in human cells or tissues comprising contacting said cells or tissues with the antisense compound of claim 51 so that the ratio of PI3K p85a to PI3K p55a is altered.

61. A method of treating a human having a disease or condition associated with PI3K signal transduction comprising administering to said human a therapeutically or prophylactically effective amount of the antisense compound of claim 1 so that PI3K signal transduction is modulated.

62. A method of treating a human having a disease or condition associated with PI3K p85 expression comprising administering to said human a therapeutically or prophylactically effective amount of the antisense compound of claim 48 so that the ratio of PI3K p85a to PI3K p50a is altered.

63. A method of treating a human having a disease or condition associated with PI3K p85 expression comprising administering to said human a therapeutically or prophylactically effective amount of the antisense compound

of claim 51 so that the ratio of PI3K p85a to PI3K p55a is altered.

ABSTRACT

5 Antisense compounds, compositions and methods are provided for modulating the expression of PI3K p85. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding PI3K p85. Methods of using these compounds for modulation of
10 PI3K p85 expression and for treatment of diseases associated with expression of PI3K p85 are provided.

SEQUENCE LISTING

<110> Brett P. Monia
Lex M. Cowser
Sue Murray
Mandy Butler
Nick Dean

<120> ANTISENSE MODULATION OF PI3K P85 EXPRESSION

<130> ISPH-0519

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Gly Tyr Gln Tyr Arg Ala Leu Tyr Asp Tyr Lys Lys Glu Arg Glu Glu
5 10 15 20

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Asp Ile Asp Leu His Leu Gly Asp Ile Leu Thr Val Asn Lys Gly Ser
25 30 35

tta gta gct ctt gga ttc agt gat gga cag gaa gcc agg cct gaa gaa 198
Leu Val Ala Leu Gly Phe Ser Asp Gly Gln Glu Ala Arg Pro Glu Glu
40 45 50

att ggc tgg tta aat ggc tat aat gaa acc aca ggg gaa agg ggg gac 246
Ile Gly Trp Leu Asn Gly Tyr Asn Glu Thr Thr Gly Glu Arg Gly Asp
55 60 65

ttt ccg gga act tac gta gaa tat att gga agg aaa aaa atc tcg cct 294
Phe Pro Gly Thr Val Glu Tyr Ile Gly Arg Lys Lys Ile Ser Pro
70 75 80

ccc aca cca aag ccc cgg cca cct cgg cct ctt cct gtt gca cca ggt 342
Pro Thr Pro Lys Pro Arg Pro Arg Pro Leu Pro Val Ala Pro Gly
85 90 95 100

tct tcg aaa act gaa gca gat gtt gaa caa caa gct ttg act ctc ccg 390
Ser Ser Lys Thr Glu Ala Asp Val Glu Gln Gln Ala Leu Thr Leu Pro
105 110 115

gat ctt gca gag cag ttt gcc cct cct gac att gcc ccg cct ctt ctt 438

Asp Leu Ala Glu Gln Phe Ala Pro Pro Asp Ile Ala Pro Pro Leu Leu			
120	125	130	
atc aag ctc gtg gaa gcc att gaa aag aaa ggt ctg gaa tgt tca act		486	
Ile Lys Leu Val Glu Ala Ile Glu Lys Lys Gly Leu Glu Cys Ser Thr			
135	140	145	
cta tac aga aca cag agc tcc agc aac ctg gca gaa tta cga cag ctt		534	
Leu Tyr Arg Thr Gln Ser Ser Asn Leu Ala Glu Leu Arg Gln Leu			
150	155	160	
ctt gat tgt gat aca ccc tcc gtg gac ttg gaa atg atc gat gtg cac		582	
Leu Asp Cys Asp Thr Pro Ser Val Asp Leu Glu Met Ile Asp Val His			
165	170	175	180
gtt ttg gct gac gct ttc aaa cgc tat ctc ctg gac tta cca aat cct		630	
Val Leu Ala Asp Ala Phe Lys Arg Tyr Leu Leu Asp Leu Pro Asn Pro			
185	190	195	
gtc att cca gca gcc gtt tac agt gaa atg att tct tta gct cca gaa		678	
Val Ile Pro Ala Ala Val Tyr Ser Glu Met Ile Ser Leu Ala Pro Glu			
200	205	210	
gta caa agc tcc gaa gaa tat att cag cta ttg aag aag ctt att agg		726	
Val Gln Ser Ser Glu Glu Tyr Ile Gln Leu Leu Lys Lys Leu Ile Arg			
215	220	225	
tcc cct agc ata cct cat cag tat tgg ctt acg ctt cag tat ttg tta		774	
Ser Pro Ser Ile Pro His Gln Tyr Trp Leu Thr Leu Gln Tyr Leu Leu			
230	235	240	
aaa cat ttc ttc aag ctc tct caa acc tcc agc aaa aat ctg ttg aat		822	
Lys His Phe Phe Lys Leu Ser Gln Thr Ser Ser Lys Asn Leu Leu Asn			
245	250	255	260
gca aga gta ctc tct gaa att ttc agc cct atg ctt ttc aga ttc tca		870	
Ala Arg Val Leu Ser Glu Ile Phe Ser Pro Met Leu Phe Arg Phe Ser			
265	270	275	
gca gcc agc tct gat aat act gaa aac ctc ata aaa gtt ata gaa att		918	
Ala Ala Ser Ser Asp Asn Thr Glu Asn Leu Ile Lys Val Ile Glu Ile			
280	285	290	
tta atc tca act gaa tgg aat gaa cga cag cct gca cca gca ctg cct		966	
Leu Ile Ser Thr Glu Trp Asn Glu Arg Gln Pro Ala Pro Ala Leu Pro			
295	300	305	
cct aaa cca cca aaa cct act act gta gcc aac aac ggt atg aat aac		1014	
Pro Lys Pro Pro Lys Pro Thr Thr Val Ala Asn Asn Gly Met Asn Asn			
310	315	320	
aat atg tcc tta caa aat gct gaa tgg tac tgg gga gat atc tcg agg		1062	
Asn Met Ser Leu Gln Ala Glu Trp Tyr Trp Gly Asp Ile Ser Arg			
325	330	335	340
gaa gaa gtg aat gaa aaa ctt cga gat aca gca gac ggg acc ttt ttg		1110	
Glu Glu Val Asn Glu Lys Leu Arg Asp Thr Ala Asp Gly Thr Phe Leu			
345	350	355	

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agg aaa ggg gga aat aac aaa tta atc aaa ata ttt cat cga gat ggg Arg Lys Gly Gly Asn Asn Lys Leu Ile Lys Ile Phe His Arg Asp Gly 375 380 385	1206
aaa tat ggc ttc tct gac cca tta acc ttc agt tct gtg gtt gaa tta Lys Tyr Gly Phe Ser Asp Pro Leu Thr Phe Ser Ser Val Val Glu Leu 390 395 400	1254
ata aac cac tac cgg aat gaa tct cta gct cag tat aat ccc aaa ttg Ile Asn His Tyr Arg Asn Glu Ser Leu Ala Gln Tyr Asn Pro Lys Leu 405 410 415 420	1302
gat gtg aaa tta ctt tat cca gta tcc aaa tac caa cag gat caa gtt Asp Val Lys Leu Tyr Pro Val Ser Lys Tyr Gln Gln Asp Gln Val 425 430 435	1350
gtc aaa gaa gat aat att gaa gct gta ggg aaa aaa tta cat gaa tat Val Lys Glu Asp Asn Ile Glu Ala Val Gly Lys Lys Leu His Glu Tyr 440 445 450	1398
aac act cag ttt caa gaa aaa agt cga gaa tat gat aga tta tat gaa Asn Thr Gln Phe Gln Glu Lys Ser Arg Glu Tyr Asp Arg Leu Tyr Glu 455 460 465	1446
gaa tat acc cgc aca tcc cag gaa atc caa atg aaa agg aca gct att Glu Tyr Thr Arg Thr Ser Gln Glu Ile Gln Met Lys Arg Thr Ala Ile 470 475 480	1494
gaa gca ttt aat gaa acc ata aaa ata ttt gaa gaa cag tgc cag acc Glu Ala Phe Asn Glu Thr Ile Lys Ile Phe Glu Glu Gln Cys Gln Thr 485 490 495 500	1542
caa gag cgg tac agc aaa gaa tac ata gaa aag ttt aaa cgt gaa ggc Gln Glu Arg Tyr Ser Lys Glu Tyr Ile Glu Lys Phe Lys Arg Glu Gly 505 510 515	1590
aat gag aaa gaa ata caa agg att atg cat aat tat gat aag ttg aag Asn Glu Lys Glu Ile Gln Arg Ile Met His Asn Tyr Asp Lys Leu Lys 520 525 530	1638
tct cga atc agt gaa att att gac agt aga aga aga ttg gaa gaa gac Ser Arg Ile Ser Glu Ile Ile Asp Ser Arg Arg Arg Leu Glu Glu Asp 535 540 545	1686
ttg aag aag cag gca gct gag tat cga gaa att gac aaa cgt atg aac Leu Lys Lys Gln Ala Ala Glu Tyr Arg Glu Ile Asp Lys Arg Met Asn 550 555 560	1734
agc att aaa cca gac ctt atc cag ctg aga aag acg aga gac caa tac Ser Ile Lys Pro Asp Leu Ile Gln Leu Arg Lys Thr Arg Asp Gln Tyr 565 570 575 580	1782
ttg atg tgg ttg act caa aaa ggt gtt cgg caa aag aag ttg aac gag Leu Met Trp Leu Thr Gln Lys Gly Val Arg Gln Lys Lys Leu Asn Glu 585 590 595	1830

tgg ttg ggc aat gaa aac act gaa gac caa tat tca ctg gtg gaa gat Trp Leu Gly Asn Glu Asn Thr Glu Asp Gln Tyr Ser Leu Val Glu Asp 600 605 610	1878
gat gaa gat ttg ccc cat cat gat gag aag aca tgg aat gtt gga agc Asp Glu Asp Leu Pro His His Asp Glu Lys Thr Trp Asn Val Gly Ser 615 620 625	1926
agc aac cga aac aaa gct gaa aac ctg ttg cga ggg aag cga gat ggc Ser Asn Arg Asn Lys Ala Glu Asn Leu Leu Arg Gly Lys Arg Asp Gly 630 635 640	1974
act ttt ctt gtc cg ^g gag agc agt aaa cag ggc tgc tat gcc tgc tct Thr Phe Leu Val Arg Glu Ser Ser Lys Gln Gly Cys Tyr Ala Cys Ser 645 650 655 660	2022
gta gtg gtg gac ggc gaa gta aag cat tgt gtc ata aac aaa aca gca Val Val Val Asp Gly Glu Val Lys His Cys Val Ile Asn Lys Thr Ala 665 670 675	2070
act ggc tat ggc ttt gcc gag ccc tat aac ttg tac agc tct ctg aaa Thr Gly Tyr Gly Phe Ala Glu Pro Tyr Asn Leu Tyr Ser Ser Leu Lys 680 685 690	2118
gaa ctg gtg cta cat tac caa cac acc tcc ctt gtg cag cac aac gac Glu Leu Val Leu His Tyr Gln His Thr Ser Leu Val Gln His Asn Asp 695 700 705	2166
tcc ctc aat gtc aca cta gcc tac cca gta tat gca cag cag agg cga Ser Leu Asn Val Thr Leu Ala Tyr Pro Val Tyr Ala Gln Gln Arg Arg 710 715 720	2214
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agtcctctg tggcagggca ctagagctgc agac atg agt gca gag ggc tac cag 595

Met Ser Ala Glu Gly Tyr Gln

1 5

tac aga gca ctg tac gac tac aag aag gag cga gag gaa gac att gac 643
Tyr Arg Ala Leu Tyr Asp Tyr Lys Lys Glu Arg Glu Glu Asp Ile Asp

19

10

15

20

cta cac ctg ggg gac ata ctg act gtg aat aaa ggc tcc tta gtg gca 691
 Leu His Leu Gly Asp Ile Leu Thr Val Asn Lys Gly Ser Leu Val Ala
 25 30 35

ctt gga ttc agt gat ggc cag gaa gcc cggt cct gaa gat att ggc tgg 739
 Leu Gly Phe Ser Asp Gly Gln Glu Ala Arg Pro Glu Asp Ile Gly Trp
 40 45 50 55

tta aat ggc tac aat gaa acc act ggg gag agg gga gac ttt cca gga 787
 Leu Asn Gly Tyr Asn Glu Thr Thr Gly Glu Arg Gly Asp Phe Pro Gly
 60 65 70

act tac gtt gaa tac att gga agg aaa aga att tca ccc cct act ccc 835
 Thr Tyr Val Glu Tyr Ile Gly Arg Lys Arg Ile Ser Pro Pro Thr Pro
 75 80 85

aag cct cgg ccc cct cga cag ctt cct gtt gct ccg ggt tct tca aaa 883
 Lys Pro Arg Pro Pro Arg Pro Leu Pro Val Ala Pro Gly Ser Ser Lys
 90 95 100

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 Thr Glu Ala Asp Thr Glu Gln Gln Ala Leu Pro Leu Pro Asp Leu Ala
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gag cag ttt gcc cct cct gat gtt gcc ccg cct ctc ctt ata aag ctc 979
 Glu Gln Phe Ala Pro Pro Asp Val Ala Pro Pro Leu Leu Ile Lys Leu
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ctg gaa gcc att gag aag aaa gga ctg gaa tgt tcg act cta tac aga 1027
 Leu Glu Ala Ile Glu Lys Lys Gly Leu Glu Cys Ser Thr Leu Tyr Arg
 140 145 150

aca caa agc tcc agc aac cct gca gaa tta cga cag ctt ctt gat tgt 1075
 Thr Gln Ser Ser Asn Pro Ala Glu Leu Arg Gln Leu Leu Asp Cys
 155 160 165

gat gcc gcg tca gtg gac ttg gag atg atc gac gta cac gtc tta gca 1123

Asp Ala Ala Ser Val Asp Leu Glu Met Ile Asp Val His Val Leu Ala			
170	175	180	
gat gct ttc aaa cgc tat ctc gcc gac tta cca aat cct gtc att cct			1171
Asp Ala Phe Lys Arg Tyr Leu Ala Asp Leu Pro Asn Pro Val Ile Pro			
185	190	195	
gta gct gtt tac aat gag atg atg tct tta gcc caa gaa cta cag agc			1219
Val Ala Val Tyr Asn Glu Met Met Ser Leu Ala Gln Glu Leu Gln Ser			
200	205	210	215
cct gaa gac tgc atc cag ctg ttg aag aag ctc att aga ttg cct aat			1267
Pro Glu Asp Cys Ile Gln Leu Leu Lys Lys Leu Ile Arg Leu Pro Asn			
220	225	230	
ata cct cat cag tgt tgg ctt acg ctt cag tat ttg ctc aag cat ttt			1315
Ile Pro His Gln Cys Trp Leu Thr Leu Gln Tyr Leu Leu Lys His Phe			
235	240	245	
tcc aag ctc tct caa gcc tcc agc aaa aac ctt ttg aat gca aga gtc			1363
Phe Lys Leu Ser Gln Ala Ser Ser Lys Asn Leu Leu Asn Ala Arg Val			
250	255	260	
ctc tct gag att ttc agc ccc gtg ctt ttc aga ttt cca gcc gcc agc			1411
Leu Ser Glu Ile Phe Ser Pro Val Leu Phe Arg Phe Pro Ala Ala Ser			
265	270	275	
tct gat aat act gaa cac ctc ata aaa gcg ata gag att tta atc tca			1459
Ser Asp Asn Thr Glu His Leu Ile Lys Ala Ile Glu Ile Leu Ile Ser			
280	285	290	295
acg gaa tgg aat gag aga cag cca gca cca gca ctg ccc ccc aaa cca			1507
Thr Glu Trp Asn Glu Arg Gln Pro Ala Pro Ala Leu Pro Pro Lys Pro			
300	305	310	
ccc aag ccc act act gta gcc aac aac agc atg aac aac aat atg tcc			1555
Pro Lys Pro Thr Thr Val Ala Asn Asn Ser Met Asn Asn Asn Met Ser			
315	320	325	

ttg cag gat gct gaa tgg tac tgg gga gac atc tca agg gaa gaa gtg			1603
Leu Gln Asp Ala Glu Trp Tyr Trp Gly Asp Ile Ser Arg Glu Glu Val			
330	335	340	
aat gaa aaa ctc cga gac act gct gat ggg acc ttt ttg gta cga gac			1651
Asn Glu Lys Leu Arg Asp Thr Ala Asp Gly Thr Phe Leu Val Arg Asp			
345	350	355	
gca tct act aaa atg cac ggc gat tac act ctt aca cct agg aaa gga			1699
Ala Ser Thr Lys Met His Gly Asp Tyr Thr Leu Thr Pro Arg Lys Gly			
360	365	370	375
gga aat aac aaa tta atc aaa atc ttt cac cgt gat gga aaa tat ggc			1747
Gly Asn Asn Lys Leu Ile Lys Ile Phe His Arg Asp Gly Lys Tyr Gly			
380	385	390	
ttc tct gat cca tta acc ttc aac tct gtg gtt gag tta ata aac cac			1795
Phe Ser Asp Pro Leu Thr Phe Asn Ser Val Val Glu Leu Ile Asn His			
395	400	405	
tac cgg aat gag tct tta gct cag tac aac ccc aag ctg gat gtg aag			1843
Tyr Arg Asn Glu Ser Leu Ala Gln Tyr Asn Pro Lys Leu Asp Val Lys			
410	415	420	
ttg ctc tac cca gtg tcc aaa tac cag cag gat caa gtt gtc aaa gaa			1891
Leu Leu Tyr Pro Val Ser Lys Tyr Gln Gln Asp Gln Val Val Lys Glu			
425	430	435	
gat aat att gaa gct gta ggg aaa aaa tta cat gaa tat aat act caa			1939
Asp Asn Ile Glu Ala Val Gly Lys Lys Leu His Glu Tyr Asn Thr Gln			
440	445	450	455
ttt caa gaa aaa agt cgg gaa tat gat aga tta tat gag gag tac acc			1987
Phe Gln Glu Lys Ser Arg Glu Tyr Asp Arg Leu Tyr Glu Glu Tyr Thr			
460	465	470	
cgt act tcc cag gaa atc caa atg aaa aga acg gct atc gaa gca ttt			2035
Arg Thr Ser Gln Glu Ile Gln Met Lys Arg Thr Ala Ile Glu Ala Phe			
475	480	485	

aat gaa acc ata aaa ata ttt gaa gaa caa tgc caa acc cag gag cgg			2083
Asn Glu Thr Ile Lys Ile Phe Glu Glu Gln Cys Gln Thr Gln Glu Arg			
490	495	500	
tac agc aaa gaa tac ata gag aag ttt aaa cgc gaa ggc aac gag aaa			2131
Tyr Ser Lys Glu Tyr Ile Glu Lys Phe Lys Arg Glu Gly Asn Glu Lys			
505	510	515	
gaa att caa agg att atg cat aac cat gat aag ctg aag tcg cgt atc			2179
Glu Ile Gln Arg Ile Met His Asn His Asp Lys Leu Lys Ser Arg Ile			
520	525	530	535
agt gag atc att gac agt agg agg ttg gaa gaa gac ttg aag aag			2227
Ser Glu Ile Ile Asp Ser Arg Arg Leu Glu Glu Asp Leu Lys Lys			
540	545	550	
cag gca gct gag tac cga gag atc gac aaa cgc atg aac agt att aag			2275
Gln Ala Ala Glu Tyr Arg Glu Ile Asp Lys Arg Met Asn Ser Ile Lys			
555	560	565	
ccg gac ctc atc cag ttg aga aag aca aga gac caa tac ttg atg tgg			2323
Pro Asp Leu Ile Gln Leu Arg Lys Thr Arg Asp Gln Tyr Leu Met Trp			
570	575	580	
ctg acg cag aaa ggt gtg cg ^g cag aag ctg aac gag tgg ctg ggg			2371
Leu Thr Gln Lys Gly Val Arg Gln Lys Lys Leu Asn Glu Trp Leu Gly			
585	590	595	
aat gaa aat acc gaa gat caa tac tcc ctg gta gaa gat gat gag gat			2419
Asn Glu Asn Thr Glu Asp Gln Tyr Ser Leu Val Glu Asp Asp Glu Asp			
600	605	610	615
ttg ccc cac cat gac gag aag acg tgg aat gtc ggg agc agc aac cga			2467
Leu Pro His His Asp Glu Lys Thr Trp Asn Val Gly Ser Ser Asn Arg			
620	625	630	
aac aaa gcg gag aac cta ttg cga ggg aag cga gac ggc act ttc ctt			2515
Asn Lys Ala Glu Asn Leu Leu Arg Gly Lys Arg Asp Gly Thr Phe Leu			

635

640

645

gtc cg_g gag agc agt aag cag ggc tgc tat gcc tgc tcc gta gtg gta 2563
 Val Arg Glu Ser Ser Lys Gln Gly Cys Tyr Ala Cys Ser Val Val

650

655

660

gac ggc gaa gtc aag cat tgc gtc att aac aag act gcc acc ggc tat 2611
 Asp Gly Glu Val Lys His Cys Val Ile Asn Lys Thr Ala Thr Gly Tyr
 665 670 675

ggc ttt gcc gag ccc tac aac ctg tac agc tcc ctg aag gag ctg gtg 2659
 Gly Phe Ala Glu Pro Tyr Asn Leu Tyr Ser Ser Leu Lys Glu Leu Val
 680 685 690 695

cta cat tat caa cac acc tcc ctc gtg cag cac aat gac tcc ctc aat 2707
 Leu His Tyr Gln His Thr Ser Leu Val Gln His Asn Asp Ser Leu Asn
 700 705 710

gtc aca cta gca tac cca gta tat gca caa cag agg cga tga 2749
 Val Thr Leu Ala Tyr Pro Val Tyr Ala Gln Gln Arg Arg *
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